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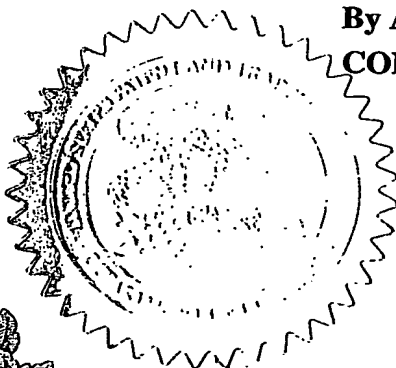
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**Title of
Invention****CYRO-PROTECTIVE AGENTS FOR MICROORGANISMS**

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Documents being submitted:	Files
us-request	ry187-usrequ.xml us-request.dtd us-request.xsl
us-fee-sheet	ry187-usfees.xml us-fee-sheet.xsl us-fee-sheet.dtd
application-body	ry187-trans.xml us-application-body.xsl application-body.dtd wipo.ent mathml2.dtd mathml2-qname-1.mod isoamsa.ent isoamsb.ent isoamsc.ent isoamsn.ent isoamso.ent isoamsr.ent isogr3.ent isomfrk.ent isomopf.ent isomscr.ent isotech.ent isobox.ent isocyr1.ent isocyr2.ent isodia.ent isolat1.ent isolat2.ent isonum.ent isopub.ent mmlextra.ent mmlalias.ent soextblx.dtd fig1.tif
Comments	

APPLICATION DATA SHEET

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Attorney Information: practitioner(s) at Customer Number: 33444  as my attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.			

Description

Cryo-Protective Agents for Microorganisms

BACKGROUND OF INVENTION

[0001] Vaccines are often produced by growing a pathogen in a culture medium, isolating the pathogen or a portion of the pathogen or a product of the pathogen and using this material as an immunogen for formulating a vaccine. Vaccines containing whole pathogens include whole cell pertussis vaccines and measles vaccines. Vaccines containing portions of the pathogen include acellular pertussis vaccines. Vaccines containing a product of the pathogen include diphtheria and tetanus vaccines. The pathogen, portion or product may require detoxification by for example chemical treatment before it can be used as a vaccine.

[0002] An example of a pathogen from which a product is used in the production of a vaccine is *Corynebacterium diphtheriae* and the product is diphtheria toxin. Diphtheria is a life-threatening disease caused by infection with *C. diphtheriae*,

a gram-positive, aerobic, rod-shaped bacterium. The disease is caused by local invasion of nasopharyngeal tissues by toxin-producing strains of *C. diphtheriae*. The organisms grow in a tough, fibrinous membrane overlying a painful, hemorrhagic, and necrotic lesion, which may be located on the tonsils or within the nasopharynx region. During typical epidemics of the past, the spread of the disease was by droplet infection. Patients who recover from diphtheria may carry toxigenic bacteria in their throats and nasopharynx for weeks or months, unless intensively treated with antibiotics.

[0003] Most of the clinical symptoms of diphtheria are due to the potent diphtheria toxin produced from corynebacteriophage carrying the *tox* gene. After the phage infects the *C. diphtheriae* strain and lysogenization has taken place, the strain becomes virulent. Toxin neutralizing antibodies (antitoxin) induced by active immunization with non-toxic forms (toxoids) of the diphtheria toxin can prevent diphtheria. The current immunization strategy is the utilization of diphtheria vaccines prepared by converting the diphtheria toxin into its non-toxic, but antigenic, toxoid form by formaldehyde treatment. The diphtheria toxoid is used in various combinations with other vaccine

components for mass immunization worldwide. The World Health Organization (WHO) recently estimated that about 100,000 cases worldwide and up to 8,000 deaths per year are due to decreased immunization of infants, waning immunity to diphtheria in adults and insufficient supply of vaccines.

[0004] The variant of the Parke Williams 8 (PW8) strain of *Corynebacterium diphtheriae* is often used to produce the exotoxin from which the toxoid is prepared by chemical modification. In general, a medium formulation with amino acids, trace vitamins, inorganic salts and a carbohydrate source such as maltose promotes excellent growth of the bacterium. Different media, such as the acid digest of casein and the enzymatic digest of beef muscle (trypsin or papain) are suitable media for toxin production. In conventional methods, the bacteria are cultivated in media containing proteinaceous material of animal origin. A commonly used medium in diphtheria production is the NZ-Amine Type A medium, which contains a casein digest. Under optimal conditions, the amount of toxin produced using NZ-Amine Type A media is 180 Lf/mL using the Limes of flocculation method.

[0005] The use of proteinaceous material of animal origin in the

production of vaccines such as the exemplified diphtheria vaccine can result in the introduction of undesirable contaminants into the diphtheria toxin produced using such a medium.

[0006] Most workers have concentrated efforts on the production of growth media substantially free or devoid of animal-components for the cultivation of pathogens such as *C. diphtheriae*. There is also a need to provide seed cultures and in particular cryoprotective agents substantially free or devoid of animal-components for microorganisms including pathogens such as *C. diphtheriae*.

SUMMARY OF INVENTION

[0007] The present invention is concerned with cryo-protective agents for microorganisms.

[0008] In one aspect of the invention, there is provided a lyophilization medium for a microorganism wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate. The lyophilization medium may comprise about 1-10% (w/v) monosodium glutamate and about 1-10% (w/v) yeast extract such as about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract. The microorganism may be a strain of bacteria including *Corynebacterium*

diphtheriae.

[0009] In a second aspect of the invention, there is provided a method for preparing a freeze-dried culture of a microorganism comprising the steps of providing a quantity of the microorganism, mixing said quantity with a lyophilization medium wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate to provide a mixture and freeze-drying said mixture. The lyophilization medium may comprise about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract such as about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract. The freeze-drying of said mixture may comprise steps of achieving a first temperature of about 30 °C for said mixture to provide a cooled mixture and maintaining said cooled mixture in a vacuum for a time until said cooled mixture is substantially dry to provide a dried mixture. Suitable vacuums are about 120 mT and suitable times are between about 10 and about 12 hours. The step of maintaining the cooled mixture in a vacuum for a time until said cooled mixture is substantially dry to provide a dried mixture may comprise maintaining said cooled mixture in a vacuum for a time of between about 10 and

about 12 hours and increasing said temperature of about 30 °C to a second temperature of about +20 °C. Suitable vacuums are about 120 mT. The microorganism may be a strain of bacteria including *Corynebacterium diphtheriae*.

[0010] There is also provided a freeze-dried lyophile comprising cells of a microorganism and a lyophilization medium wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate. The lyophilization medium may comprise about 1–10% (w/v) monosodium glutamate and about 1–10% (w/v) yeast extract such as about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract. The microorganism may be a strain of bacteria including *Corynebacterium diphtheriae*.

BRIEF DESCRIPTION OF DRAWINGS

[0011] The present invention will be further understood from the following description with reference to the drawing, in which: Figure 1 shows a flow diagram outlining the preparation and lyophilization of a *C. diphtheriae* culture.

DETAILED DESCRIPTION

[0012] A flow diagram outlining the preparation and lyophilization of *C. diphtheriae* culture is shown in Figure 1. A

lyophile of *C. diphtheriae* strain 1M1514N3S was inoculated onto an agar plate containing Phytone™ peptone agar and incubated at 36°C for 43–48 hours. The composition of Phytone™ peptone medium is described in Tables 1–2 below.

[0013] Table 1. Composition of the Phytone™ peptone medium containing 15 g/L of Phytone™

Ingredient	Quantity per Liter
Phytone™ Peptone	15 g
Acetic acid	7.2 mL
Maltose	25 g
Growth Factors	8 mL
10% L-Cystine	2 mL
60% Sodium Lactate	1.7 mL
PH	7.5

Table 2. Composition of the growth factor solution

Ingredient	Quantity
Magnesium sulphate	225 g
Beta Alanine	2.30 g
Pimelic acid	0.15 g
Zinc sulphate	0.80 g
Copper sulphate	0.50 g
Manganese chloride	0.24 g
Nicotinic acid	4.6 g
Hydrochloric acid, concentrated	30 mL
Water for Injection	1000 mL

Table 3. A typical analysis of Phytone™ Peptone as provided by the manufacturer Difco Laboratories is provided below:

Nitrogen Content/Physical Characteristics	
Total Nitrogen (TN) (%)	9.0
Amino Nitrogen (AN) (%)	2.4
AN/TN	0.27
Ash (%)	12.4
Loss on Drying (%)	1.5
NaCl (%)	4.0
pH (2% solution)	7.1
Elemental Analysis	
Calcium (µg/g)	1001
Magnesium (µg/g)	2435
Potassium (µg/g)	31547
Sodium (µg/g)	34037
Chloride (%)	0.76
Sulfate (%)	0.67
Phosphate (%)	0.64
Amino Acid Analysis	
Free	Total

Alanine (%)0.32.6Aspartic Acid (%)0.33.9Glutamic Acid
 (%)0.35.9Histidine (%)0.20.8Leucine (%)0.82.3Methionine
 (%)0.20.2Proline (%)0.11.8Threonine (%)0.10.5Tyrosine
 (%)0.20.8Arginine (%)0.62.1Cystine (%)0.4Destroyed by
 hydrolysisGlycine (%)0.21.5Isoleucine (%)0.21.3Lysine
 (%)1.22.4Phenylalanine (%)0.21.4Serine
 (%)0.40.5Tryptophan (%)Below level of detectionDestroyed
 by hydrolysisValine (%)0.11.5The culture was resuspended
 in 5 mL of Phytone™ peptone medium and 1.5 mL of the
 culture transferred to a primary shake flask containing 90
 mL of Phytone™ peptone medium containing 0.9 mL of a
 1:10 diluted phosphate solution (32% (w/v)) and 0.45 mL
 of 1:2 diluted calcium chloride solution (53 % (w/v)). The
 culture was incubated at 36°C, 200 rpm for 24 hours. Five
 mL of the culture was transferred to a secondary shake
 flask culture containing 250 mL of Phytone™ peptone
 medium containing 2.5 mL of a 1:10 diluted phosphate
 solution (32 % (w/v)) and 1.25 mL of a 1:2 calcium chlo-
 ride_Toc518533307_Toc784736 solution (53 % (w/v)).
 The culture was incubated at 36°C for a further 24–28
 hours. Ten mL of the above secondary shake flask culture
 was dispensed into five 50 mL sterile screw capped cen-
 trifuge tubes and centrifuged at 6 000 xg for 10 minutes

at 4°C.

[0014] The supernatant was decanted and the pellet of each tube, re-suspended in 5 mL of one of the following lyophilization media: a) 10% (w/v) skim milk (Animal Control) b) 10% (w/v) yeast extract c) 10% (w/v) Phytone™ peptone d) 5% (w/v) monosodium glutamate + 10% (w/v) yeast extract e) 10% (w/v) Phytone™ peptone + 10% (w/v) yeast extract + 0.25% (w/v) agar. The cultures in the above lyophilization medium were dispensed in 0.25 mL amounts in 1 mL glass vials and freeze dried as follows.

[0015] *Freeze-Drying cycle* The product temperature was allowed to reach 30 °C and held at that temperature for about 10–12 hours under a vacuum of 120 mT. After 10–12 hours, the product temperature was increased and maintained at 20 °C under a vacuum of 120 mT. The vials are sealed under vacuum and stored at 4°C. The freeze dried cultures were analyzed for viability by measuring colony forming units (CFU/mL) on Columbia blood agar plates.

[0016] _Toc518533307_Toc784736 The results of CFUs obtained before and after freeze-drying for *C. diphtheriae* strain are shown tabulated in Tables 4, 5, 6 and 7.

[0017] Table 4: Comparison of CFU counts of the freeze dried cultures of *C. diphtheriae* in skim milk and animal compo-

nent-free lyophilization medium. The CFU count before
 freeze-drying of *C. diphtheriae* was 6.0×10^9 CFU/
 mL Lyophilization medium CFU /mL % Viability Skim Milk
 (Animal Component Control) 1.24×10^9 21 MSG + Yeast
 extract 1.08×10^9 18 Table 5: Comparison of CFU counts of
 the freeze dried cultures of *C. diphtheriae* in skim milk and
 animal component-free lyophilization medium as a func-
 tion of time. (*C. diphtheriae* strain) Lyophilization medium-
 Day 0 Day 7 Day 16 Day 45 Day 86 Day 120 Skim Milk $1.24 \times$
 10^9 5.6×10^7 7×10^6 3×10^6 3×10^6 3×10^8 MSG + Yeast Ex-
 tract 1.08×10^9 9.5×10^8 3.2×10^8 7.9×10^8 3.5×10^8 $3.4 \times$
 10^8 Table 6: Screening of the animal component-free
 lyophilization medium and their respective CFU counts in
 comparison to animal component lyophilization medium
 after freeze-drying.

[0018] Freezing Mixture CFU /mL Skim Milk (animal component)
 1.2×10^7 10% Yeast extract 1.9×10^7 10% Phy-
 tone™ peptone 1.36×10^8 MSG + Yeast extract 6.0×10
 8 Yeast extract + Phytone™ peptone + Agar 1.76×10^8 Table
 7: Comparison of CFU counts of the freeze dried cultures
 of *C. diphtheriae* in animal component and animal compo-
 nent-free lyophilization medium.

[0019] CFU/mL Time (Days) 10% Skim Milk 5% MSG + 10% YE 02.04 x

10^9 1.0 x 10^9 12.0 x 10^7 1.22 x 10^9 165 x 10^6 2.96 x 10^8
 452.0 x 10^6 1.02 x 10^9 862.0 x 10^6 3.2 x 10^8 The most sta-
 ble mixture for freeze-drying is the mixture of Yeast ex-
 tract (10% w/v) with mono sodium glutamate (5%w/v), as
 shown in Tables 4-7

Claims

[c1] We claim:

1.A lyophilization medium for a microorganism wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate.

[c2] 2.The lyophilization medium of claim 1, comprising about 1–10% (w/v) monosodium glutamate and about 1–10% (w/v) yeast extract.

[c3] 3.The lyophilization medium of claim 2, comprising about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract.

[c4] 4.The lyophilization medium of claim 1 or 2 or 3 wherein the microorganism is a strain of bacteria.

[c5] 5.The lyophilization medium of claim 4 wherein the strain of bacteria is *Corynebacterium diphtheriae*

6.A method for preparing a freeze-dried culture of a microorganism comprising the steps of:

providing a quantity of the microorganism;

mixing said quantity with a lyophilization medium

wherein the medium is substantially free of animal-de-

rived products and comprises yeast extract and monosodium glutamate to provide a mixture; and freeze-drying said mixture.

- [c6] 7.The method of claim 4, wherein the lyophilization medium of comprises about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract.
- [c7] 8.The method of claim 5, wherein the lyophilization medium of comprises about 1–10% (w/v) monosodium glutamate and about 1–10% (w/v) yeast extract.
- [c8] 9.The method of claim 6 or 7 or 8 wherein freeze-drying of said mixture comprises steps of:
 - (a)achieving a first temperature of about 30 °C for said mixture to provide a cooled mixture;
 - (b)maintaining said cooled mixture in a vacuum for a time until said cooled mixture is substantially dry to provide a dried mixture.
- [c9] 10.The method of claim 7 wherein the vacuum is about 120 mT.
- [c10] 11.The method of claim 8 wherein the time is between about 10 and about 12 hours.
- [c11] 12.The method of claim 7 wherein the step of maintaining said cooled mixture in a vacuum for a time until said

cooled mixture is substantially dry to provide a dried mixture comprises:

- (a) maintaining said cooled mixture in a vacuum for a time of between about 10 and about 12 hours; and
- (b) increasing said temperature of about 30 °C to a second temperature of about +20 °C.

[c12] 13. The method of claim 10 wherein the vacuum is about 120 mT.

[c13] 14. The method of claim 6 or 7 or 8 wherein the microorganism is a strain of bacteria.

[c14] 15. The method of claim 14 wherein the strain of bacteria is a strain of *Corynebacterium diphtheriae*

16. A free-dried lyophile comprising cells of a microorganism and a lyophilization medium wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate.

[c15] 17. The freeze-dried lyophile of claim 12, wherein the medium comprises about 1–10% (w/v) monosodium glutamate and about 1–10% (w/v) yeast extract.

[c16] 18. The freeze-dried lyophile of claim 13, wherein the medium comprises about 5 % (w/v) monosodium glutamate and about 10% (w/v) yeast extract.

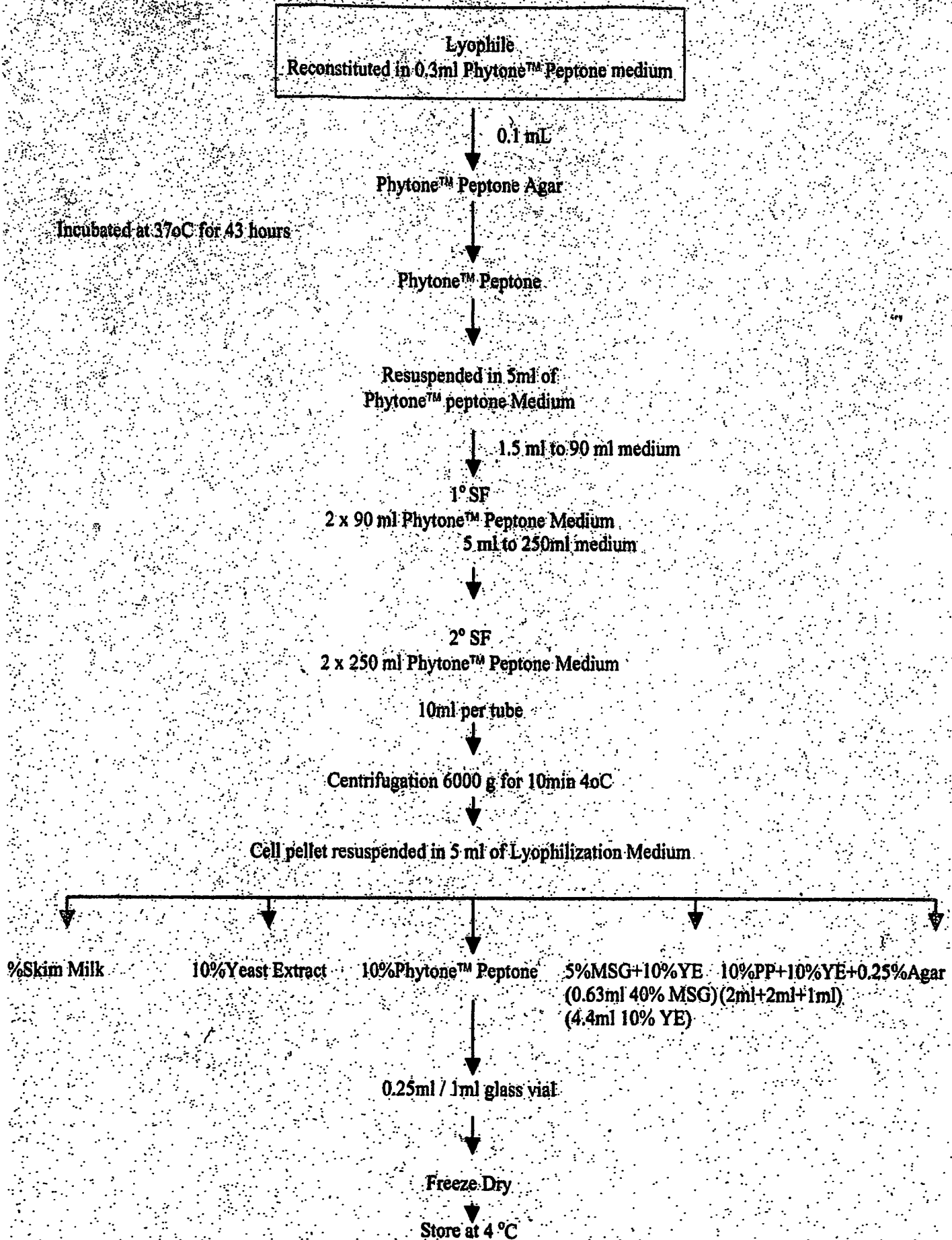
[c17] 19. The freeze-dried lyophile of claim 16 or 17 or 18 wherein the microorganism is a strain of bacteria.

[c18] 20. The freeze-dried lyophile of claim 19 wherein the strain of bacteria is a strain of *Corynebacterium diphtheriae*.

Cryo-Protective Agents for Microorganisms

Abstract

A lyophilization medium for a microorganism is provided wherein the medium is substantially free of animal-derived products and comprises yeast extract and monosodium glutamate. The lyophilization medium can be used for cryoprotection of strains of bacteria such as *Corynebacterium diphtheriae*. Method for preparing a freeze-dried culture of a microorganism using the lyophilization medium, and lyophiles of microorganisms are also provided.

Figure 1: A flow diagram outlining the preparation and lyophilization of a *C. diphtheriae* culture.

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